(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 078 636
B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: 13.04.88

(51) Int. Cl.^{4}: G 01 N 27/30, G 01 N 33/48

(21) Application number: 82305597.5

(22) Date of filing: 21.10.82

(54) Sensor for components of a liquid mixture.

(30) Priority: 23.10.81 GB 8132034

(43) Date of publication of application:
11.05.83 Bulletin 83/19

(45) Publication of the grant of the patent:
13.04.88 Bulletin 88/15

(84) Designated Contracting States:
BE CH DE FR GB IT LI NL SE

(56) References cited:
DD-A- 154 041
US-A-4 144 143
ANALYTICAL CHEMISTRY, vol. 51, no. 1, January 1979 T. SHINBO et al. "Potentiometric enzyme electrode for lactate", pages 100-101
Journal of Electrochem.Soc., 123(9)1334-1339
Journal of Organometallic Chemistry, 149(1978) pages 231-244 Biotechnology Letters. Vol. 3 no.4, pages 187-192 (1981)

The file contains technical information submitted after the application was filed and not included in this specification

(73) Proprietor: Genetics International, Inc.
128 Sidney Street
Cambridge Massachusetts 02139 (US)

(73) Proprietor: Higgins, Irving John
Cotswold Graze Hill
Ravensden Bedford MK44 2TF (GB)

(72) Inventor: Higgins, Irving John
Lion Fields Barford Road
Wilden Bedfordshire (GB)
Inventor: Hill, Hugh Allen Oliver
9 Clover Close
Oxford (GB)
Inventor: Plotkin, Elliot Verne
14 George Street
Bedford Bedfordshire (GB)

(74) Representative: Clifford, Frederick Alan et al
MARKS & CLERK 57/60 Lincoln's Inn Fields
London WC2A 3LS (GB)

EP 0 078 636 B1

Courier Press, Leamington Spa, England.

Description

This invention relates to an electrode, a method of assay for detecting the presence of, measuring the amount of, and/or monitoring the level of one or more selected components in a liquid mixture and to the use of a ferrocene or ferrocene derivative in said assay as well as to a ferrocene or ferrocene derivative for use as a mediator in an *in vivo* assay.

While use may be made of this invention in chemical industry, especially where complex mixtures are encountered (e.g. in food chemistry or biochemical engineering) it is of particular value in biological investigation and control techniques. More particularly, it lends itself to animal or human medicine, and in particular to *in vivo* measuring or monitoring of components in body fluids.

For convenience, the invention will be described with reference primarily to one such procedure, the determination of glucose in a diabetic human subject, by the use of equipment which, while usable on a specific or occasional basis also lends itself to temporary or permanent implantation. However, while the provision of an implantable glucose sensor is a major object of the invention, other and broader objects are not hereby excluded.

In vivo glucose sensors have already been proposed. One proposal is based on direct oxidation of glucose at a catalytic platinum electrode (see Hormone and Metabolic Research, Supplement Series No. 8, pp 10—12 (1979)) but suffers from the drawback of being non-specific and of being easily poisoned by interfering substances. Another proposal, for a procedure more specific to glucose, involves the use of glucose oxidase on an oxygen electrode (Adv. Exp. Med. Biol, *50* pp 189—197 (1974)) but is not very responsive to the high glucose concentrations. Other systems using glucose oxidase have been proposed but not fully investigated for *in vivo* methods, see e.g. J. Solid-Phase Biochem. *4* pp 253—262 (1979).

The inventors have recently carried out *in vitro* studies of enzyme-catalyzed reactions using a mediator in solution to transfer the electrons arising from the enzyme, during its action, directly to the electrode, as described in Biotechnology Letters *3* pp 187—192 (1981).

It has now been realised that mediator compounds can be associated with the sensor electrode structure thus rendering such electrodes available for use by *in vivo* methods.

In one aspect the present invention consists in a sensor electrode for use in liquid mixtures of components for detecting the presence of, measuring the amount of, and/or monitoring the level of, one or more components in a liquid mixture, by sensing flow of current at the electrode when an oxidoreductase enzyme catalyses a redox reaction of a substrate and a mediator compound transfers electrons between the electrode and the catalytically active oxidoreductase enzyme, the electrode being characterised in that it comprises the mediator compound and the oxidoreductase enzyme at least at an external surface of electrically conductive material of the electrode, and the electrode being further characterised in that the mediator compound is a ferrocene compound.

The use of a ferrocene compound to transfer charge between an electrode and a biological molecule is known in itself.

For example, the chapter by Szentrimay, Yeh and Kuwana in Electrochemical Studies of Biological Systems, D. Sawyer (Ed), Chapter 6, page 143 (Am Chem Soc. Wash DC (1977) reviews determination of electrochemical characteristics of oxidoreductase enzymes and like substances using a technique known as the Indirect Coulometric Titration ("ICT"). In the ICT method, an enzyme is itself titrated with electrons, giving enforced conversion between reduced and oxidised forms of the enzyme, in the absence of substrate. By the use of an optically transparent electrode, spectral information such as UV absorbance is acquired during the titration.

Correlation of the electrochemical and spectral ICT data then allows determination of physical properties of the enzyme itself, including the stoichiometry designated "*n*", and the energetics designated "E°'". Szentrimay describes the use of various compounds in solution to facilitate the transfer of electrons between the electrode and the enzyme. Such compounds for use in the ICT method are termed mediator-titrants, and include viologens and ferrocene compounds.

The work on ferrocene compounds as ICT mediator-titrants is reviewed by Szentrimay *et al.* They note the limited solubility of some ferrocene derivatives, which is contrary to their criteria for "ideal" mediator-titrants. Reference is made to an article by Yeh and Kuwana in J. Electrochem Soc (1976) *123*, 1334, which describes how solubilization can be achieved, using a micelle-solubilized ferrocene as a mediator-titrant in the ICT determination of *n* and E°'.

An article by Shinbo and Sugiura, Analytical Chemistry (1979) *51*, 100 is concerned with a potentiometric method for determination of biological redox molecules. More specifically, Shinbo describes a potentiometric enzyme electrode for determination of lactate. The electrode is of relatively complicated construction which includes a membrane to separate two aqueous systems containing ferrocyanide and ferricyanide. The membrane itself contains dibutylferrocene, and the enzyme lactate dehydrogenase is present in the outer aqueous system. When the ratio of ferricyanide to ferrocyanide is altered by a catalytic reaction on one side of the membrane, the resulting potential difference is sensed.

The enzyme catalysed oxidation of ferrocene and some substituted ferrocenes by hydrogen peroxide in the presence of peroxidase has been described by Epton *et al.* in J. Organometallic Chem (1978) *149*, 231. In passing, this article refers to the Yeh and Kuwana article discussed above.

US Patent 4144143 describes the production of a methyl viologen film on an electrode, typically a minigrid electrode. The methyl viologen serves as a mediator to transfer electrons from the electrode to large biological molecules, such as heme proteins, spinach ferrodoxin, or components of the mammalian phosphorylation sequence. The patent has an example of an assay of the heme proteins myoglobin and hemoglobin using the electrode.

A method of assay is also provided by this invention. Thus, in a further aspect, this invention provides a method of assay for detecting the presence of, measuring the amount of, and/or monitoring the level of, one or more components in a liquid mixture. In the method, a redox reaction of a substrate is catalysed by an oxidoreductase enzyme at an external surface of electrically conductive material of an electrode, a mediator compound is employed at the surface of the electrode to transfer electrons between the oxidoreductase enzyme and the electrode during the redox reaction, and the current generated by the transfer of electrons is detected using the electrode. The mediator compound is a ferrocene compound.

In a further aspect, this invention is directed specifically to the use of a ferrocene compound as a mediator compound in an assay of a component in a liquid system by the steps of catalysing a redox reaction of the component with an oxidoreductase enzyme at an external surface of electrically conductive material of the electrode, employing a mediator compound at the surface of the electrode to transfer electrons between the oxidoreductase enzyme and the electrode during the redox reaction, and detecting the transfer of electrons using the electrode.

To this end, the invention also provides a ferrocene compound, for use as a mediator compound in an *in vivo* diagnostic assay in a liquid system by the steps of catalysing a redox reaction of a substrate with an oxidoreductase enzyme at an external surface of electrically conductive material of an electrode, employing a mediator compound at the surface to transfer electrons between the oxidoreductase enzyme and the electrode during the redox reaction, and detecting the transfer of electrons using the electrode.

Preferably the electrode is designed to determine glucose *in vivo*. The enzyme is therefore preferably a glucose oxidase, or possibly a glucose dehydrogenase, for example a bacterial glucose dehydrogenase.

Glucose oxidase (β-D-glucose:oxygen oxidoreductase, of enzyme classification EC 1.1.3.4) is a well known type of enzyme. Bacterial glucose dehydrogenase (EC 1.1.99.17) is a more recent discovery, and is believed to be a quinoprotein with a polycyclicquinone prosthetic group (PQQ). Reference is made to Duine et al TIBS, (Oct. 1981) 278—280 and Arch. Microbial (1982) 131.27—31.

Use of such a bacterial glucose dehydrogenase in the present invention has certain advantages over the use of a glucose oxidase. The major advantage is that it can give an oxygen-insensitive glucose sensor, since the enzyme does not use oxygen as an electron acceptor. A suitable enzyme can be purified (as described in more detail below) either by conventional chromatographic techniques or by two-phase aqueous partition from a range of micro-organisms. A preferred micro-organism is *Acinetobacter calcoaceticus* but various *Gluconobacter* species (e.g. *Gluconobacter oxidans*) or *Pseudomonas* species (e.g. *Pseudomonas fluorescens, Pseudomonas aeruginosa)* can also be used.

Mediator compounds which may be used in accordance with the invention possess the electron-transfer property referred to above.

The mediator compound is a ferrocene or ferrocene derivative.

A ferrocene has, as its fundamental structure, an iron atom held "sandwiched" by dative bonds between two cyclopentadienyl rings. It is an electroactive organometallic compound, acting as a pH-independent reversible one-electron donor. Various derivatives are available (e.g. with various substituents on the ring structure, possibly in polymer form) differing in redox potential, aqueous solubility and binding constant to glucose oxidase or bacterial glucose dehydrogenase enzyme.

For instance, the redox potential of the parent compound is +422 mV vs NHE. By introducing functional groups on to the ring system E'° can be varied between +300 and +650 mV. Moreover, the water-solubility of the carboxyl-substituted ferrocenes is greater than that of the parent compound. Further description will be found in Szentrimay, 1977, ACS Symposium Series, *38*, 143—169, see page 154.

Among specific mediator compounds of this type are ferrocene itself, 1,1'-ferrocene dicarboxylic acid, and dimethyl ferrocene.

The electrically conductive material of the electrode itself can be a metal, or carbon either as a pre-formed rod or as an electrode shape made up from a paste of carbon particles. Surface condition of the electrode is usually important. If metal, the surface can be roughened where it contacts the active materials (enzyme and/or mediator). If solid carbon, the surface can be "oxidised" i.e. previously heat-treated in an oven with oxygen access.

Certain combinations of the above-materials, and certain configurations of electrode, are preferable in practice.

In a particularly valuable form of the invention, the electrode comprises a carbon core, a layer of ferrocene or a ferrocene derivative at a surface thereof and a layer of glucose oxidase or glucose dehydrogenase at the surface of the ferrocene layer. The enzyme layer is preferably immobilised at the surface of the underlying mediator, retained in a self sustaining gel layer thereupon and/or has a retention layer thereover permeable to the glucose molecule.

The carbon core can itself be solid or a stiff

paste of particles. Normally, it will present a smooth surface for the ferrocene or ferrocene derivative, which may be adhered thereto in a number of ways, for example, for a monomeric ferrocene or ferrocene derivative, by deposition from a solution in a readily evaporatable liquid, e.g. an organic solvent such as toluene.

The enzyme to be coated on the ferrocene or ferrocene derivative can be the glucose oxidase or the bacterial glucose dehydrogenase. The glucose oxidase can be immobilised to the underlying surface e.g. by the carbodiimide material DCC (1-cyclohexyl-3-(2-morpholino ethyl) carbo-diimide metho-*p*-toluene sulphonate) which gives a thin strongly bound layer, a good linear response to low glucose concentrations, and oxygen insensitivity (because of the competition from the ferrocene with oxygen for electrons transferred to the enzyme redox centre from the substrate). Using DCC immobilisation of glucose oxidase on ferrocene also extends the top end of the linear range of the sensor from about 2 mM to 40 mM.

Other methods of immobilisation, or other forms of protection e.g. incorporation into a self-supporting gelatine layer, are also possible.

The bacterial glucose dehydrogenase can also be immobilised at the mediator surface, but may be merely deposited from an evaporatable solution, or held in a gelatin layer.

Optionally, but preferably when being used on live blood, a protective membrane surrounds both the enzyme and the mediator layers, permeable to water and glucose molecules. This can be a film of dialysis membrane, resiliently held e.g. by an elastic O-ring. It can however also with advantage be a layer of cellulose acetate, e.g. as formed by dipping the electrode into a cellulose acetate solution in acetone.

It will be apparent that while the invention has primary relevance to a sensor electrode, especially such an electrode specific for glucose, it also relates to the combination of such an electrode and temporary or permanent implantation means, e.g. a needle-like probe. Also, such an electrode, connected or connectable, with signal or control equipment, more especially with an insulin administration means, constitutes an aspect of the invention.

The electrodes according to the invention permit the manufacture of an improved macro-sensor for use in hospital analytical glucose-sensing instruments of the existing type. The advantages compared to known instruments would be that the increased linear range together with very low oxygen sensitivity would allow omission of the dilution step involved in blood analysis in current instruments. Moreover, as described in more detail below, the response times of such electrodes are short (24—36 seconds) for 95% of steady state depending on complexity of solution).

The electrodes of the invention, on the macro-scale could be incorporated into simple, cheap electronic digital read-out instruments for doctors surgeries or diabetic home-testing kits.

Use of a small version of the macro-sensor would be possible in a device which automatically takes a blood sample from the finger, brings it into contact with the sensor, amplifies the signal and gives a digital readout. Use of a micro-version of the sensor in a watch type device for monitoring glucose interstitial fluid in the skin could also be envisaged. It would be worn on the wrist and would have a disposable sensor cartridge in the back with one or more separate, fines, needle-type sensors. Each would feed into the electronics which is several sensors were used would cross-refer the current inputs to ensure reliability.

Connection of such devices to external insulin delivery systems could act as a feedback control loop for an insulin pump. Indeed, such a device could be housed in the canula used to feed insulin into the body from a pump and again serve as a sensor for the feedback loop. Other uses such as hypoglycaemia alarm, or digital read-out monitor, are also possible.

The invention will be further described with reference to Examples 6—12 and to the accompanying drawings, in which:

Figure 1 is a diagrammatic longitudinal cross-section through a glucose sensor electrode,

Figure 2 is a diagrammatic longitudinal cross-section through a different form of glucose sensor electrode,

Figure 3 is a graph of the current sensed by the electrode of Figure 2, against glucose concentration,

Figure 4 is a diagrammatic longitudinal cross-section of the electrode of Figure 2 located within a hypodermic needle,

Figure 5 is a diagrammatic longitudinal cross-section through a yet further glucose sensor electrode,

Figure 6 is a graph analogous to Figure 3 for the electrode of Figure 5,

Figure 7 is a graph analogous to Figure 3 for an electrode incorporating a glucose dehydrogenase.

Example 1

Purification of Quinoprotein Glucose Dehydrogenase (GDH) from *Acinetobacter calcoaceticus*

(a) Growth or organisms

Strain NCTC 7844 was grown on sodium succinate (20 gl^{-1}) in batch culture at pH 8.5 and 20°C. Cells were harvested after 20 hours (A_{600}=6.0) using a Sharples centrifuge, and stored frozen.

(b) Purification of glucose dehydrogenase

The method is based on the method of J A Duine et al (Arch Microbiol, 1982 vide supra) but with modifications as follows.

1. 100 g. of cells were thawed, resuspended in 3 300 ml. of 56 mM Tris/39 mM glycine and treated for 20 minutes at room temperature with 60 mg lysozyme.

2. Triton X-100 extracts were combined and treated with 0.01 $mgml^{-1}$ of deoxyribonuclease I for 15 minutes at room temperature. The resulting suspension was then centrifuged at 48000 xg for 25 minutes at 4°C. The supernatant from this

centrifugation was then treated with ammonium sulphate. The yellow protein precipitating between 55 and 70% ammonium sulphate was resuspended in 36 mM Tris/39 mM glycine containing 1% Triton X-100 and dialysed against that buffer at 4°C for 5 hours.

3. Active fractions from the CM sepharose CL-6B column were combined and concentrated using Millipore CX-30 immersible ultrafilters.

Example 2
Purification of Quinoprotein Glucose Dehydrogenase from *Acinetobacter calcoaceticus* (alternative method)

(a) Growth of organisms
The method of Example 1 was repeated.

(b) Purification of GDH
The method is based on the partitioning of proteins between two liquid phases. The steps were:—

1. Cells were thawed and resuspended at 3 ml/g wet weight in 50 mM sodium phosphate, pH 2,0. They were then pre-cooled on ice and passed once through a Stansted pressure cell (made by Stansted Fluid Power Ltd., Stansted, Essex, UK) at 25000 psi (about 1723 bar). This provided the cell-free extract.

2. The cell-free extract was then mixed for 15 minutes at room temperature with 50% (w/v) polyethylene-glycol · 1000, 50% (w/v) sodium phosphate, pH 7.0 and distilled water in the proportions of 2:4:3:1 respectively. This mixture was centrifuged at 5000 rpm for 5 minutes to break the emulsion.

3. The lower layer was aspirated off and desalted immediately, by either diafiltration using an Amicon hollow-fibre ultrafiltration cartridge of 10000 mwt cut off, or by passage through a Sephadex G50 (medium grade) gel filtration column.

4. The resulting solution was concentrated using an Amicon PMIO membrane in a nitrogen pressure cell.

Example 3
Interaction between ferrocene and glucose oxidase

DC cyclic voltammetry was used to investigate the homogeneous kinetics of the reaction between ferrocene and the glucose oxidase enzyme under substrate excess conditions. A two compartment electrochemical cell of 1.0 ml volume fitted with a Luggin capillary was used. The cell contained a 4.0 mm gold disc working electrode, a platinum gauze counter-electrode and a saturated calomel electrode as a reference. A series of voltammograms for ferrocene was recorded at scan rates of 1—1000 mVs^{-1} in 50 mM potassium phosphate buffer, pH 7.0. The data showed that the mediator acted as a reversible, one-electron acceptor $E° = +165$ mV vs. SCE.

Addition of 50 mM glucose has no discernable effect on the electrochemistry of the mediator (50 µM). Upon addition of glucose oxidase (10 µM), however, an enhanced anodic current was observed in the voltammogram at oxidising potentials with respect to the mediator. This indicated catalytic regeneration of the reduced form of the mediator by glucose oxidase. Quantitative kinetic data was obtained for this reaction using an established procedure (Nicholson, R. S. and Shain, I., 1964, *Anal Chem., 36, 707)*. The mediator gave a second order rate constant for the reaction between ferricinium ion and reduced glucose oxidase of $K = 10^4$ mol $^{-1}s^{-1}$. This ability of the ferricinium ion to act as a rapid oxidant for glucose oxidase facilities the efficient coupling of the enzymic oxidation of glucose.

Example 4
The procedure of Example 3 was repeated using 1,1'-ferrocene dicarboxylic acid instead of ferrocene. The value of E_o' was determined to be +420 mV, and the second order rate constant of the ferricinium ion and reduced glucose oxidase was again $10^4 mol^{-1}s^{-1}$, thus confirming the conclusions drawn from Example 3.

Example 5
Glucose oxidase/polyviologen

For experimental purposes an *in vitro* sensor was made up as shown in Figure 1. This Example employs polyviologen as mediator, but serves to illustrate construction of a sensor electrode.

A silver disc 1 was glued at 2 over the lower end of a length of 12 mm glass tubing 3. A wire 5 was soldered to the back of the silver disc at 6. The tubing was placed inside a "Teflon" sleeve 7, and the outside of the disc 1 roughened at 8. A solution containing glucose oxidase and the o-dibromxylene/4,4'-bipyridyl polyviologen was applied over the roughened surface 8 and dried to layer 9. A subsequent layer 10 of molten agar also containing the glucose oxidase and polyviologen, of approximately 1 mm in thickness was placed over the layer 9, and solidified. Finally, dialysis membrane 11 was placed over the assembly and held by O-ring 12.

To demonstrate the principle of using the polyviologen mediator to couple electrically the glucose oxidase to an electrode, the sensor was placed in a buffered electrochemical cell, which was stirred and agitated with a current of nitrogen. The electrode was held at −90 mV vs. SCE, and current flow measured on a chart recorder. Aliquots of glucose were added. As the glucose concentration in the solution increased, over the range of 1 to 8 mM, the current also increased, indicating that the electrode was acting as a glucose sensor.

Example 6
Glucose/Oxidase Dimethyl Ferrocene
Mini electrode for *in vivo* glucose sensing in skin

A graphite rod 13 (Figure 2) with an oxidised surface, 30 mm long×0.9 mm diameter is glued with epoxy resin into a nylon tube 14.25 mm long, 0.9 mm inside diameter, 1.3 mm outside dia-

meter. The end 15 of the electrode is dipped into a solution of dimethyl ferrocene, (10 mg/ml) in toluene, and the solvent is then allowed to evaporate.

The end 15 of the electrode is placed into a solution of water soluble DCC (25 mg/ml) in acetate buffer, pH 4.5 for 1 hour. It is then rinsed, in buffer only, for 5 minutes and thereafter placed in a solution of glucose oxidase (10 mg/ml) in acetate buffer, pH 5.5, for 1½ hours before again rinsing in buffer. The tip of the electrode 15, with the layers of dimethyl ferrocene and immobilised enzyme is then dipped into a solution of cellulose acetate dissolved in acetone and formamide and put into ice water for several minutes, to give a protected and stable electrode.

This electrode was connected to a potentiostat, together with a suitable counter electrode and calomel reference electrode and placed in a solution containing glucose. The potential of the working electrode is kept at +100 mV to 300 mV relative to the calomel electrode, i.e. as low as possible to avoid oxidation of potentially interfering substances. A current is produced which is proportional to the glucose concentration. The time for 95% of response is less than 1 minute and the electrode gives a near linear response over the range 0—32 mM glucose, as shown in Figure 3. Slow loss of activity ferrocene (due to slow loss of ferricinium ion) can be minimised by keeping the electrode at a potential between 0 and −100 mV vs. a standard calomel electrode when not in use.

Figure 4 shows in section an electrode structure in which an electrode (references as in Figure 2) of much smaller size is held within a hypodermic needle 16 plugged at its point 17 but with side windows 18 for passage of blood or other body fluid. The small size of such an electrode and its linear response over a large range of glucose concentrations makes it possible to use the electrode for *in vivo* glucose determination on both severely diabetic and normal individuals.

Example 7

Glucose Oxidase/Ferrocene

In vitro sensor

A carbon rod 19 (Figure 5) Ultra carbon, grade U5, 6 mm×15 mm) with a metal connector 20 secured in one end was sealed in glass tubing 21 (borosilicate, 6 mm i.d.×mm) with an epoxy resin (araldite), (not shown). The exposed surface at 22 was polished with emery paper and washed with distilled water. The entire rod was heated in an oven for 40 h at 200°C to give an oxidised surface at 22.

15 µl of ferrocene (20 mg/ml in toluene) was pipetted onto the oxidised surface and allowed to dry completely. The rod was then placed in 1 ml of water-soluble DCC (25 mg/ml in 0.1 M acetate buffer, pH 4.5) for 80 min at room temperature. The rod was then washed in 0.2 M carbonate buffer, pH 9.5 and placed in a glucose oxidase solution (Sigma type X, 12.5 mg/ml) for 1½ hours at room temperature. It was finally washed with water with a pH 7 buffer containing 0.2 g/l glucose and stored at 4°C.

The characteristics of the above electrode were determined in a nitrogen-saturated buffer solution (0.2 M sodium phosphate, pH 7.3) and are shown in Figure 6. The curve is linear from 2 to 25 mM glucose and reaches saturation current at 100 mM in glucose.

In separate tests with an air-saturated buffer at 8 mM glucose the current was measured as being at least 95% of that produced in the nitrogen-saturated buffer.

Response time was also measured, being the time taken to achieve 95% of maximum current for the given glucose concentration. With the nitrogen-saturated buffer an electrode as described above had a response time of 24 seconds at 2 mM glucose and 60 seconds at 6 mM glucose. With the same buffer, such an electrode modified by a cellulose acetate membrane coating (produced as in Example 7) gave response times of 36 seconds (2 mM) and 72 seconds (6 mm). With blood, this modified electrode gave response times of 36 seconds (blood with a known 2 mM glucose content) and 72 seconds (blood at a known 6 mM glucose content).

Electrodes as above were stored in 20 mM sodium phosphate pH 7 for 4 weeks at 4°C as a stability test and thereafter re-examined as above. The results were within 10% and usually within 5% of results with a freshly made electrode.

Example 8

Glucose Dehydrogenase/Ferrocene

A stiff carbon paste was made up from 1.6 g of Durco activated charcoal and 2.5 ml of liquid paraffin. A Pasteur pipette for 6 mm internal diameter was blocked 2 mm from its wide end by a silver disc to which a connecting wire was soldered. The space between the disc and the end of the pipette was filled with the carbon paste, and the surface of the paste was polished with paper until smooth and even.

A single 20 microlitre drop of a toluene solution of ferrocene (20 mg/l) was placed on the smooth surface and allow to spread and evaporate to leave a film of the ferrocene.

A further drop of 25 microlitres of bacterial glucose dehydrogenase solution as obtained in Example 1, containing between 1 and 10 mg. of protein per ml, was placed on this ferrocene surface and allowed to spread.

A cover of dialysis membrane was secured over the so-coated end of the electrode by a tight-fitting O-ring.

Example 9

Glucose Dehydrogenanse/Ferrocene

The procedure of Example 8 was repeated but using an electrode the same carbon paste packed into the spaced defined between the end of a length of nylon tubing and a stainless steel hypodermic needle shaft inserted therein terminating 2 mm. short of the tubing end, so as to define a small electrode body. The electrode was

further fabricated using only 5 microlitres of the ferrocene solution and 1 microlitre of the enzyme solution.

Example 10
Glucose Dehydrogenase/Ferrocene

The procedure of Example 8 was repeated using as electrode a solid carbon rod (Ultracarbon grade U5 6 mm diameter) within a Pyrex glass tube 3 cm long and 6 mm internal diameter and connected to a stainless steel hypodermic shaft, giving a construction similar to that shown in Figure 5. The end of the carbon rod was polished smooth with emery cloth and aluminium oxide powder prior to the application of the ferrocene solution.

Example 11
Glucose Dehydrogenase/Ferrocene

A gelation-entrapped glucose dehydrogenase was prepared by mixing at 37°C, 25 mg gelatin, 0.5 ml of the glucose dehydrogenase solution as described in Example 8 and 2.5 microlitres of TEMED. After complete dissolving of the gelatin 200 microlitres of the solution was spread over an area of 2 cm^2 and allowed to dry under a stream of cold air.

A disc of 0.25 cm^2 area was then used instead of the drop of enzyme solution in Example 8.

Example 12
Glucose Dehydrogenase/Ferrocene

Example 11 was repeated using a disc of the gel of 1 mm^2 area and applying it instead of the drops of enzyme solution in the construction of Example 9.

The results obtained from the electrode described in Examples 8 to 12 are all similar, and show a very specific electrode of low oxygen sensitivity. By way of example, the electrode of Example 11 was calibrated and gave the results shown in Figure 7.

Devices such as shown in the Examples 6—12 offer advantages over most of the enzyme-based sensors currently available. When compared to such sensors prior to dilution steps, the present electrode has an equal or faster response time, the ability to operate under anaerobic conditions, greater oxygen insensitivity (important in blood samples, where oxygen concentration is variable), extended linear range covering the complete physiological range and comparable specificity, stability and ease of manufacture.

Claims

1. A sensor electrode (13, 14, 15) for use in detecting the presence of, measuring the amount of, and/or monitoring the level of, one or more components in a liquid mixture, by monitoring the flow of current at the electrode when an oxidoreductase enzyme catalyses a redox reaction of a substrate and a mediator compound transfers electrons between the electrode and the catalytically active oxidoreductase enzyme, the electrode being characterised in that it comprises the mediator compound and the oxidoreductase enzyme at least at an external surface (15) of electrically conductive material of the electrode the material being suitable for carrying the said flow of current, and the electrode being further characterised in that the mediator compound is a ferrocene or a ferrocene derivative.

2. A sensor electrode as claimed in claim 1 characterised in that the enzyme catalyses a reaction of glucose whereby there is provided a glucose sensor.

3. A sensor electrode as claimed in claim 2, characterised in that the enzyme is a glucose oxidase.

4. A sensor electrode as claimed in claim 2, characterised in that the enzyme is a bacterial glucose dehydrogenase.

5. A sensor electrode as claimed in claim 4, characterised in that the glucose dehydrogenase is separated from *Acinetobacter calcoaceticus*.

6. A sensor electrode as claimed in any of claims 1 to 5 characterised in that the mediator is ferrocene itself, 1,1'-ferrocene-dicarboxylic acid, or dimethyl ferrocene.

7. A sensor as claimed in any of claims 1 to 6 characterised in that the said electrically conductive material of the electrode is a carbon particle paste or solid carbon.

8. A sensor electrode as claimed in any of claims 1 to 7 characterised by the particular combination in which the electrically conductive material is carbon (13), the mediator compound is a layer of ferrocene or a ferrocene derivative and the enzyme is a glucose oxidase or bacterial glucose dehydrogenase located upon the layer of mediator compound (15).

9. A sensor electrode as claimed in claim 8 characterised in that the ferrocene or ferrocene derivative is in monomeric form and deposited from a readily evaporable organic solvent therefor.

10. A sensor electrode as claimed in any of claims 8 to 1 characterised by having an outermost protective membrane (11) permeable to water and glucose molecules.

11. A sensor electrode as claimed in any one of the preceding claims 1 to 10 in combination with temporary or permanent implantation means such as a needle-like probe (16).

12. A sensor electrode combination as claimed in claim 11 in further combination with signal or control equipment.

13. A method of assay, excluding assay methods of treatment or diagnosis performed on the human or animal body, for detecting the presence of, measuring the amount of, and/or monitoring the level of, one or more components in a liquid mixture, wherein a redox reaction of a substrate is catalysed by an oxidoreductase enzyme forming part of an electrode at an external surface of electrically conductive material of the electrode, a ferrocene or ferrocene derivative as mediator compound is employed at the surface of the electrode forming a part thereof to transfer

electrons between the oxidoreductase enzyme and the electrode during the redox reaction, and the current generated by the transfer of electrons is monitored using the electrode.

14. The use of a ferrocene or ferrocene derivative as a mediator compound in an assay, excluding assay methods of treatment or diagnosis performed on the human or animal body, in a liquid system by the steps of catalysing a redox reaction of a substrate with an oxidoreductase enzyme forming part of an electrode at an external surface of electrically conductive material of the electrode, employing a mediator compound at the surface of the electrode forming a part thereof to transfer electrons between the oxidoreductase enzyme and the electrode during the redox reaction, and monitoring the flow of current at the electrode.

15. A ferrocene or ferrocene derivative, for use as a mediator compound in an *in vivo* diagnostic assay in a liquid system by the steps of catalysing a redox reaction of a substrate with an oxidoreductase enzyme forming part of an electrode at an external surface of electrically conductive material of the electrode, employing the mediator compound also forming part of the electrode at the electrode surface to transfer electrons between the oxidoreductase enzyme and the electrode during the redox reaction, and monitoring the flow of current at the electrode.

Patentansprüche

1. Sensorelektrode (13, 14, 15) zur Verwendung für den Nachweis des Vorliegens von und der Messung der Menge an und/oder der Überwachung des Gehaltes von einer oder mehreren Komponenten in einem flüssigen Gemisch durch Überwachung des Stromflusses an der Elektrode, wenn ein Oxidoreductaseenzym eine Redoxreaktion eines Substrats katalysiert und eine Mediatorverbindung Elektronen zwischen der Elektrode und dem katalytisch aktiven Oxidoreductaseenzym überträgt wobei die Elektrode dadurch gekennzeichnet ist, daß sie die Mediatorverbindung und das Oxidoreductaseenzym an wenigstens einer äußeren Oberfläche (15) von elektrisch leitfähigem Material der Elektrode enthält, wobei das Material sich zur Leitung dieses Stromflusses eignet, und die Elektrode sich weiter dadurch auszeichnet, daß die Mediatorverbindung ein Ferrocen oder ein Ferrocenderivat ist.

2. Sensorelektrode nach Anspruch 1, dadurch gekennzeichnet, daß das Enzym eine Reaktion von Glucose katalysiert, wodurch ein Glucosesensor geliefert wird.

3. Sensorelektrode nach Anspruch 2, dadurch gekennzeichnet, daß das Enzym eine Glucoseoxidase ist.

4. Sensorelektrode nach Anspruch 2, dadurch gekennzeichnet, daß das Enzym eine bakterielle Glucosedehydrogenase ist.

5. Sensorelektrode nach Anspruch 4, dadurch gekennzeichnet, daß die Glucosedehydrogenase aus Acinetobacter calcoaceticus abgetrennt ist.

6. Sensorelektrode nach irgendeinem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß der Mediator Ferrocen selbst oder 1,1'-Ferrocendicarbonsäure oder Dimethylferrocen ist.

7. Sensor nach irgendeinem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß das elektrisch leitfähige Material der Elektrode eine Paste aus Kohlenstoffteilchen oder fester Kohlenstoff ist.

8. Sensorelektrode nach irgendeinem der Ansprüche 1 bis 7, gekennzeichnet durch die besondere Kombination, in welcher das elektrisch leitfähige Material Kohlenstoff (13) ist, die Mediatorverbindung eine Schicht aus Ferrocen oder einem Ferrocenderivat ist und das Enzym eine Glucoseoxidase oder ein bakterielle Glucosedehydrogenase ist, die auf der Schicht von Mediatorverbindung (15) liegt.

9. Sensorelektrode nach Anspruch 8, dadurch gekennzeichnet, daß das Ferrocen oder Ferrocenderivat in monomer Form vorliegt und aus einem leicht verdampfbaren organischen Lösungsmittel dafür abgeschieden ist.

10. Sensorelektrode nach irgendeinem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß sie eine zuäußerst liegende Schutzmembran (11) aufweist, die für Wasser und Glucosemoleküle durchlässig ist.

11. Sensorelektrode nach irgendeinem der Ansprüche 1 bis 10 in Kombination mit temporären oder permanenten Implantationsmitteln, wie einer nadelähnlichen Sonde (16).

12. Sensorelektrodenkombination nach Anspruch 11 in weiterer Kombination mit Signal- oder Kontrolleinrichtung.

13. Assayverfahren, ausgenommen Assayverfahren zur Behandlung oder Diagnose, die am menschlichen oder tierischen Körper durchgeführt werden, zum Nachweis der Vorliegens von, Messung der Menge an und/oder Überwachung des Gehaltes an einer oder mehreren Komponenten in einem flüssigen Gemisch, wobei eine Redoxreaktion eines Substrats durch ein Oxidoreductaseenzym katalysiert wird, das Teil einer Elektrode an einer äußeren Oberfläche von elektrisch leitfähigem Material der Elektrode bildet, ein Ferrocen oder Ferrocenderivat als Mediatorverbindung an der Oberfläche der Elektrode verwendet wird und einen Teil davon bildet, um Elektronen zwischen dem Oxioreductaseenzym und der Elektrode während der Redoxreaktion zu überführen und der durch die Überführung der Elektronen erzeugte Strom unter Verwendung der Elektrode überwacht wird.

14. Verwendung eines Ferrocens oder eines Ferrocenderivates als Mediatorverbindung in einem Assay, ausgenommen Assayverfahren zur Behandlung oder Diagnose, die am menschlichen oder tierischen Körper durchgeführt werden, in einem flüssigen System durch die Stufen der Katalyse einer Redoxreaktion eines Substrates mit einem Oxidoreductaseenzym, das Teil einer Elektrode an einer äußeren Oberfläche von elektrisch leitfähigem Material der Elektrode bildet, unter Verwendung einer Mediatorverbindung an der Oberfläche der Elektrode unter Bildung eines

Teils davon zur Überführung von Elektronen zwischen dem Oxidoreductaseenzym und der Elektrode während der Redoxreaktion und Überwachung des Stromflusses an der Elektrode.

15. Ferrocen oder Ferrocenderivat zur Verwendung als Mediatorverbindung in einem in vivo Diagnoseassay in einem flüssigen System durch die Stufen der Katalyse einer Redoxreaktion einer Substanz mit einem Oxidoreductaseenzym, das Teil einer Elektrode an einer äußeren Oberfläche von elektrisch leitfähigem Material der Elektrode bildet, wobei die verwendete Mediatorverbindung ebenfalls einen Teil der Elektrode an der Elektrodenoberfläche bildet, um Elektronen zwischen dem Oxidoreductaseenzym und der Elektrode während der Redoxreaktion zu überführen, und der Stromfluß an der Elektrode überwacht wird.

Revendications

1. Electrode de détection (13, 14, 15) utilisee pour détecter la présence, mesurer la quantité, et/ou contrôler la teneur, d'un ou de plusieurs constituants d'un mélange liquide, en contrôlant le débit de courant dans l'électrode lorsqu'une enzyme oxydoréductase catalyse une réaction d'oxydoréduction d'un substrat, et qu'un composé médiateur transfère des électrons entre l'électrode et l'oxydoréductase active de façon catalytique,

électrode caractérisée en ce qu'elle comporte le composé médiateur et l'oxydoréductase au moins sur une surface externe (15) de la matière conductrice d'électricité de l'électrode, cette matière étant adaptée pour véhiculer ledit débit de courant, et en ce que le composé médiateur est le ferrocène ou un dérivé de ferrocène.

2. Electrode de détection selon la revendication 1, caractérisée en ce que l'enzyme catalyse une réaction du glucose afin de réaliser un détecteur de glucose.

3. Electrode de détection selon la revendication 2, caractérisée en ce que l'enzyme est une glucose-oxydase.

4. Electrode de détection selon la revendication 2, caractérisée en ce que l'enzyme est une glucose-déshydrogénase bactérienne.

5. Electrode de détection selon la revendication 4, caractérisée en ce que la glucose-deshydrogénase est obtenue à partir *d'Acinetobacter calcoaceticus*.

6. Electrode de détection selon l'une quelconque des revendications 1 à 5, caractérisée en ce que le médiateur est le ferrocène lui-même, l'acide (1,1') ferrocène-dicarboxylique ou le diméthyl ferrocène.

7. Détecteur selon l'une quelconque des revendications 1 à 6, caractérisé en ce que ladite matière conductrice d'électricité de l'électrode est une pâte de particules de carbone ou du carbone solide.

8. Electrode de détection selon l'une quelconque des revendications 1 à 7, caractérisée par la combinaison particulière dans laquelle la matière conductrice d'électricité est du carbone (13), le composé médiateur est une couche de ferrocène ou d'un dérivé de ferrocène et l'enzyme est une glucose-oxydase ou une glucose-deshydrogenase bactérienne située sur la couche de composé médiateur (15).

9. Electrode de détection selon la revendication 8, caractérisée en ce que le ferrocène ou le dérivé de ferrocène est sous forme monomère et est en conséquence, déposé à partir d'un solvant organique évaporable rapidement.

10. Electrode de détection selon l'une quelconque des revendications 8 à 9, caractérisé en ce qu'elle possède une membrane protectrice (11) la plus à l'extérieur, perméable aux molécules d'eau et de glucose.

11. Electrode de détection selon l'une quelconque des revendications 1 à 10 en combinaison avec des moyens d'implantation temporaire ou permanent tels qu'une sonde en forme d'aiguille (16).

12. Combinaison d'électrode de détection selon la revendication 11, combinée en outre avec un équipement de signal et de commande.

13. Procédé de test, excluant les procédés de test de traitement ou de diagnostic réalisé sur le corps humain ou animal, pour détecter la présence, mesurer la quantité, et/ou contrôler la teneur d'un ou de plusieurs constituants d'un mélange liquide, dans lequel une réaction d'oxydoréduction d'un substrat est catalysée par une oxydoréductase, formant une partie d'une électrode située à la surface externe d'une matière conductrice d'électricité de l'électrode, un ferrocène ou un dérive de ferrocène est employé comme composé médiateur à la surface de l'électrode en formant une partie de celle-ci, afin de transférer les électrons entre l'enzyme oxydoréductase et l'électrode durant la réaction d'oxydoréduction, et le courant généré par le transfert d'électrons est contrôlé en utilisant l'électrode.

14. Utilisation du ferrocène ou d'un dérivé du ferrocène comme composé médiateur dans un test, à l'exception des procédés de test de traitement ou de diagnostic réalisés sur le corps humain ou animal, dans un système liquide comprenant les étapes:

—de catalyse d'une réaction d'oxydoréduction d'un substrat avec une oxydoréductase formant une partie d'une électrode au niveau de la surface externe d'une matière conductrice d'électricité de l'électrode,

—d'emploi d'un composé médiateur à la surface de l'électrode formant une partie de celle-ci, afin de transférer les électrons entre l'oxydoréductase et l'électrode durant la réaction d'oxydoréduction,

—et de contrôle du débit de courant au niveau de l'électrode.

15. Ferrocène ou dérivé de ferrocène utilisé en tant que composé médiateur dans un test de diagnostic in vivo dans un système liquide caractérisé par les étapes:

—de catalyse d'une réaction d'oxydoréduction d'un substrat avec une enzyme oxydoréductase,

formant une partie d'une électrode au niveau d'une surface externe d'une matière conductrice d'électricité de l'électrode,

—d'emploi d'un composé médiateur formant aussi une partie de l'électrode, à la surface de l'électrode, afin de tranférer les électrons entre l'oxydoréductase et l'électrode durant la réaction d'oxydoréduction et,

—de contrôle du débit de courant au niveau de l'électrode.

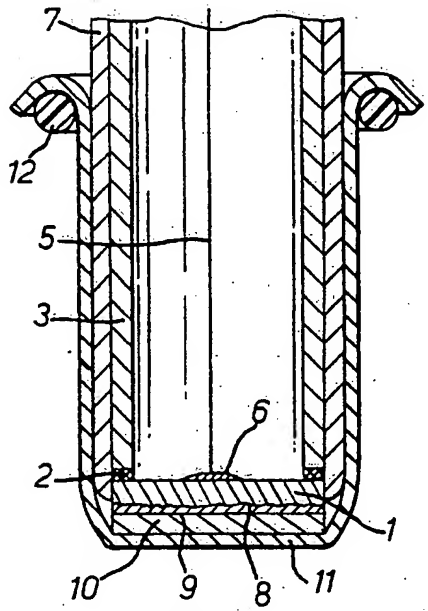


FIG. 1.

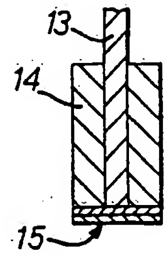


FIG.2.

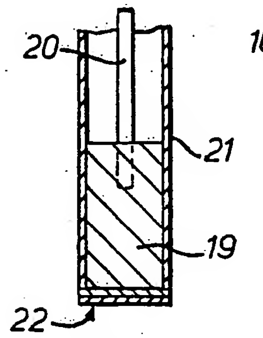


FIG.5.

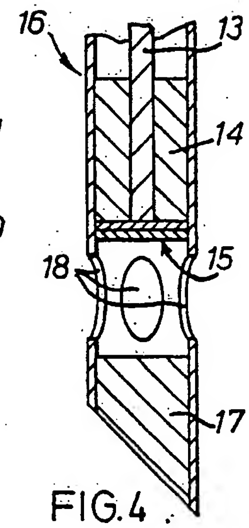


FIG.4.

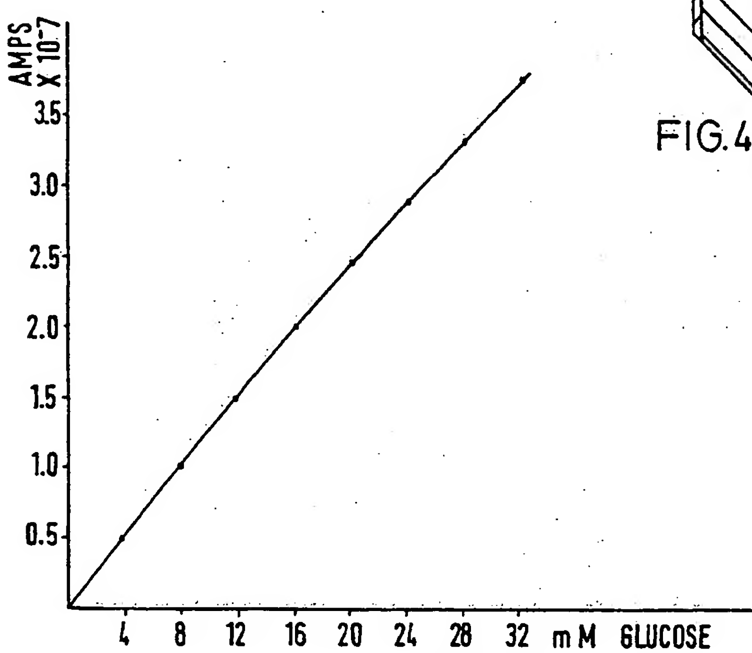
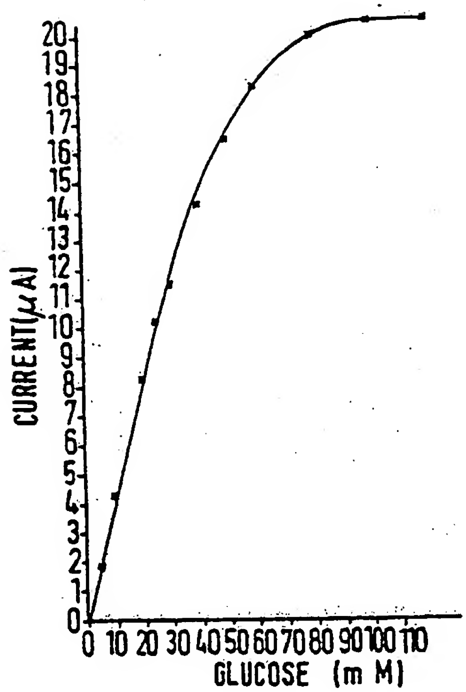
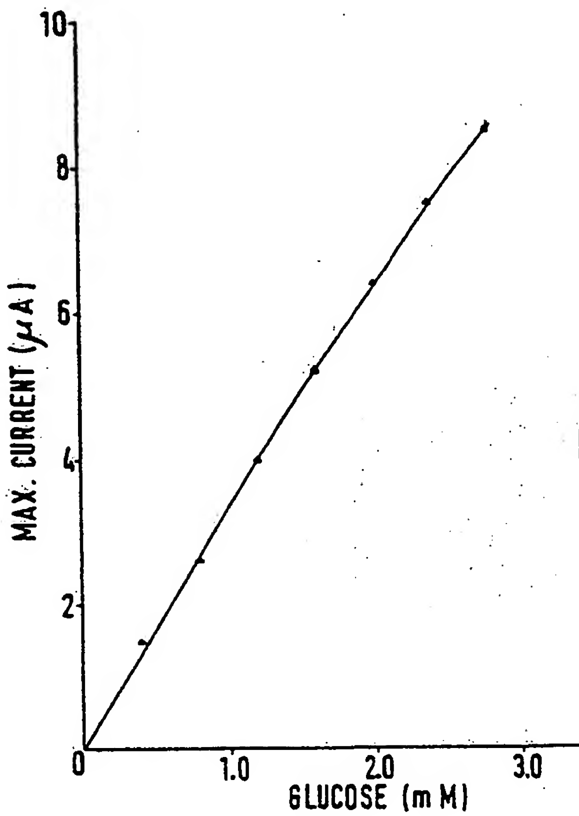


FIG.3.



CURRENT(μA)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
0 10 20 30 40 50 60 70 80 90 100 110
GLUCOSE (m M)

FIG.6.



MAX. CURRENT (μA)
10
8
6
4
2
0
1.0
2.0
3.0
GLUCOSE (m M)

FIG.7.